

**The structure of Arabidopsis NPR1: its function as a
salicylic acid receptor and a metal-binding protein**

By

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Abstract

The Arabidopsis NPR1 protein regulates systemic acquired resistance dependent on salicylic acid. Analyses by plant two-hybrid analysis in vivo and pull-down assays in vitro showed that the BTB/POZ domain of NPR1 at the N-terminus serves as an autoinhibitory domain to negate the function of the transactivation domain at the C-terminus through direct binding of these two domains. It was also shown that the binding of the BTB/POZ domain to the C-terminus of NPR1 was abolished by SA treatment, suggesting that SA could interfere directly with this binding. By gel filtration, it was demonstrated that SA affects the conformation of full-length NPR1, confirming the role of NPR1 as an SA receptor. Gel filtration analysis also indicated that NPR1 could be converted from an oligomer to a dimer with SA treatment. Furthermore, one N-terminal deletion $\Delta 513$ has been shown to act as a metal-binding protein and its two Cys-521 and Cys-529 are important for binding to Ni^{2+} by pull-down assays.

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List of abbreviations

2,4-D -- 2,4-dichlorophenoxyacetic acid

AA -- amino acid

Ala -- alanine

as-1 -- activation sequence 1

Avr -- avirulence

BA2H -- benzoic acid-2-hydroxylase

BTB/POZ -- Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger

BTH -- benzothiadiazole S-methylester

bZIP -- basic domain/leucine zipper

CaMV -- cauliflower mosaic virus

CCS -- copper chaperone for SOD1

CT -- C-terminus

Cys -- cysteine

DB -- DNA binding domain

DIR1 -- defective in resistance 1

DTH9 -- detachment 9

DTT -- dithiothreitol

E. coli -- *Escherichia coli*

EDS1 -- enhanced disease susceptibility 1

EDS5 -- enhanced disease susceptibility 5

ET -- ethylene

GAL -- galactosidase

Gal -- Galactose

GAL4 DB -- GAL4 DNA-binding domain

GFP -- green fluorescent protein

GH3 -- Glycoside Hydrolase

GSTF8 -- *glutathione S-transferase F8*

HA -- hemagglutinin

HABA -- 2-4'-hydroxy-benzeneazobenzoic acid

His – Histidine
 HR – hypersensitive response
 ICS – isochorismate synthase
 ICS1 – isochorismate synthase 1
 INA – 2, 6-dichloroisonicotinic acid
 IPL – isochorismate pyruvate lyase
 JA – jasmonic acid
 Leu – leucine
 LTP – lipid transfer proteins
 Mar – multiple antibiotic resistance
 MES – methyl esterase
 MeSA – methyl salicylate
 MeSAG – methyl salicylate *O*- β -glucoside
 NahG – salicylate hydroxylase
 NIM1 – non-inducible immunity 1
 NIMIN – NIM1-interacting
 Ni-NTA – nickel-nitrilotriacetic acid
 NLS – nuclear localization sequence
 NMR – Nuclear magnetic resonance
 NPR1 – non-expressor of pathogenesis related genes 1
 NT – N-terminus
 PAD4 – phytoalexin deficient 4
 PAGE – polyacrylamide gel electrophoresis
 PAL – phenylalanine ammonia lyase
PR – Pathogenesis-related
 R – resistance
 ROS – reactive oxygen species
 SA – salicylic acid
 SABP2 – salicylic acid-binding protein 2
 SAG – SA *O*- β -glucoside

SAGT – SA glucosyltransferase
 SAMT – SA methyltransferase
 SAR – systemic acquired resistance
 SDS – sodium dodecyl sulphate
 SFD1 – suppressor of fatty acid desaturase deficiency1
 SGE – salicyloyl glucose ester
 SID2 – salicylic acid-induction deficiency 2
sin1 – suppressor of *npr1-1*, inducible 1
 SNI1 – suppressor of NPR1-1, inducible 1
 SOD1-- Cu/Zn superoxide dismutase
 SPR – surface plasmon resonance
 TA – transactivation domain
 TF – transcription factor
 TGA – TGACG motif binding
 UAS – upstream activation sequence
Vir – *virulence*
 WHY1 – whirly 1
 ZID – zinc finger protein with interaction domain

Introduction

The *Arabidopsis* Nonexpressor of Pathogenesis-Related gene 1 (NPR1) has been identified as a master regulator of *Pathogenesis-Related (PR)* gene activation. It has been demonstrated that the signal molecule salicylic acid (SA) accumulates in plant cells upon pathogen attack, and that it is mandatory for the deployment of a systemic broad-spectrum plant disease resistance termed systemic acquired resistance (SAR) (Durrant and Dong, 2004). Despite the numerous published studies on SA, the mechanism by which SA is involved in the activation of *PR* genes is not well understood. NPR1 exists as an oligomer held by intermolecular disulfide bonds in the cytosol. Upon SA treatment, it is reduced to a monomer which enters the nucleus and activates the *PR* genes (Tada et al., 2008). Furthermore, it has been found that the coactivator function of NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines in the transactivation domain (TA) (Rochon et al., 2006). However, in the absence of SA, full-length NPR1 is not able to transactivate, while the TA domain is able to transactivate in the presence and absence of SA. From this study, we can speculate that the BTB/POZ domain is autoinhibitory. Furthermore, these results raise the possibility that the BTB/POZ domain may inhibit the transactivation domain of the CT of NPR1 by directly binding to it. The purpose of this project is therefore to investigate whether the N-terminus (NT) of NPR1 interacts with its C-terminus (CT) in the absence of salicylic acid (SA), thereby functioning as an inhibitory domain to mask the transactivation domain in the CT of NPR1. In addition to that, the speculations that the addition of SA breaks the interaction

between NT and CT of NPR1 exposing the transactivation domain of the CT, and that NPR1 may function as a metal-binding protein are also explored in more detail. The focus of the study is to develop methods to assay the structure of NPR1, and particularly to assess the interaction between the NT and CT of NPR1. The methods used include plant two-hybrid assays, pull-down assays, and chromatography to explore the effect of SA on NPR1, and also the metal-binding capacity of NPR1.

Chapter 1. Literature Review

1.1 Systemic acquired resistance in plant

Plants have developed complex defense mechanisms in response to various types of stresses. It has been found that transcription factors induced by stresses are important in inducing defense systems against the stresses (Nishizaw, et al., 2006). These stresses could be salicylic acid, auxin, hydrogen peroxide, and also biotic stimuli such as a pathogen attack.

Pathogens carry avirulence genes (Avr) which encodes proteins recognized by specific receptor proteins in plant cells encoded by host resistance \otimes genes. Resistance-gene-mediated recognition of pathogens by plants typically results in a hypersensitive response (HR), which is characterized by rapid apoptotic-like cell death in the local region surrounding the infection site. The HR restricts the growth and spread of pathogens to other parts of plants, and is accompanied by the induction of local and systemic expression of a number of defense genes, ultimately leading to the development of a broad-range long-lasting resistance to pathogen infection known as systemic acquired resistance (SAR) (Després et al., 2003; Kumar and Klessig, 2003; Verk et al., 2008). Therefore, SAR is a general plant resistance response that can be induced during a local infection by an avirulent pathogen. Concurrent with SAR development, SA and pathogenesis-related (*PR*) gene transcripts also accumulate in plants. Because of the correlation between *PR* gene expression and systemic resistance, these *PR* genes are the best-characterized NPR1-dependent SA-induced genes, and are considered excellent markers for SAR. The *PR-1* gene is a member of

a family of *PR* genes, and is the most common molecular marker used to measure the induction of SAR.

A necessary prerequisite for the deployment of SAR and *PR*-gene activation is the accumulation of the mandatory metabolite salicylic acid (SA). Treatment of plant tissues with exogenous SA or its functional analogs BTH (benzothiadiazole S-methylester) and INA (2, 6-dichloroisonicotinic acid) is also sufficient for *PR*-gene induction and the establishment of SAR, in a process referred to as chemical SAR (Cao *et al.*, 1994; Ward *et al.*, 1991).

1.2. *NPR1 acting as a central regulator of SAR*

NPR1 protein, also known as Non-inducible Immunity1 (NIM1) is recognized as the key positive regulator of *PR-1* gene expression and SAR because of the analysis showing that SA-mediated *PR* gene expression and SAR are impaired in *npr1* mutant plants while disease resistance is enhanced in plants overexpressing NPR1 (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook *et al.*, 1996). Moreover, NPR1 is an important component of SA signaling, and it functions downstream of SA in the defense signaling pathway (Shah, 2003; Kumar and Klessig, 2003). However, the observation that in *npr1* mutant plants, the establishment of induced systemic resistance (ISR) by non-pathogenic root rhizobacteria is blocked suggests that the *npr1* mutant is not only deficient in SA-mediated defense, but that NPR1 plays a role in other defense signaling pathways dependent on the signaling molecules jasmonic acid (JA) and ethylene (ET) (Pieterse *et al.*, 1998).

In resting cells, the endogenous NPR1 protein is localized to both the nucleus and the cytosol (Després *et al.*, 2000). Other studies suggest that NPR1 is located in the cytosol, where it plays a role in an antagonistic cross-talk between the SA and JA-mediated pathways through a mechanism that is not well understood, possibly by preventing the production of positive regulators of JA signaling or facilitating the delivery of negative regulators of JA signaling to the nucleus (Spoel *et al.*, 2003). In the absence of SA treatment or pathogen attack, NPR1 is maintained in the cytoplasm as an oligomer formed through intermolecular disulfide bonds. Nevertheless, this NPR1 oligomerization was determined when NPR1 is fused with the green fluorescence protein (GFP) (NPR1-GFP). The oligomerization hence could be due to self-assembly of GFP but not NPR1. Upon treatment with SA or its analog or pathogen infection, a redox change is triggered, thereby reducing NPR1. Monomerization of NPR1 is promoted, presumably as a result of the reduction of an intermolecular disulfide bridge between Cys-82 and Cys-216 of NPR1. These monomeric forms of NPR1 are then translocated to the nucleus with the help of NPR1 nuclear localization sequences. Nuclear localization is a prerequisite for *PR1* gene activation (Mou *et al.*, 2003; Kinkema *et al.*, 2000). It was also found that S-nitrosylation of NPR1 by S-nitrosoglutathione (GSNO) at Cys-156 facilitates its oligomerization in the presence of SA. Conversely, the SA-induced NPR1 oligomer-to-monomer reaction is catalyzed by thioredoxins (TRX) (Tada *et al.*, 2008). The regulation of NPR1 through the opposing action of GSNO and TRX and involving redox changes suggests a new mechanism by which NPR1 enters the

nucleus to activate *PR* genes and SAR. Durrant and Dong (2004) found that in response to SA, NPR1 moves to the nucleus where it interacts with the TGA class of basic leucine zipper transcription factors to induce defense gene expression, thereby activating SAR.

NPR1 acting as a cofactor interacts with members of the TGA family of basic/leucine zipper (bZIP) transcription factors that bind the *activation sequence-1* (*as-1*) or *as-1*-like sequences identified in the promoter of *PR-1* genes (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000). The *cis* element *activation sequence-1*, originally identified as promoter regulatory elements of plant viruses, such as the cauliflower mosaic virus (CaMV) 35S promoter, and agrobacterial pathogens, has been found in numerous stress-responsive plant promoters. For instance, it is found in the pathogen-inducible *Arabidopsis thaliana* *Pathogenesis Related-1* (*PR-1*) gene and is activated in the course of a defense response upon pathogen attack. In addition, it is also activated by salicylic acid (SA), auxin and its analog 2, 4-D (Lebel et al., 1998). Nowadays, a large number of studies are focusing on NPR1/TGA factors/*as-1*-like elements since they are interconnected and important in disease resistance. However, some *as-1*-containing genes, such as *glutathione S-transferase F8* (*GSTF8*) are expressed in an NPR1-independent manner (Blanco et al., 2005), but these NPR1-independent pathways are not well understood.

In addition to members of the TGA family of basic domain/leucine zipper transcription factors, NIMIN (NIM1-interacting) proteins, especially NIMIN1, have also been found to interact with NPR1 in the presence of SAR inducers. These act as

transcriptional repressor to negatively regulate NPR1 and further down-regulate NPR1-dependent genes, thus compromising *PR* gene expression and SAR (Weigel et al., 2005). This negative regulation of function of NPR1 by NIMIN proteins has provided a mechanism to modulate specific features of SAR such as the tradeoff occurring between SA- and JA-dependent pathways. Li and colleagues reported the isolation and characterization of *snl1* (suppressor of *npr1-1*, inducible 1), a mutant that restores SA- and INA-inducible *PR* gene expression and disease resistance, suggesting that SN11 is also a negative regulator of SAR (Li et al., 1999). Recently, Chern demonstrated another strong repressor of SAR named NRR (for Negative Regulator of disease Resistance) identified from rice. NRR is found to display its suppression of *PR* gene expression and SAR by binding to NPR1. Suppression is dependent on its putative repression domain (Chern et al., 2008).

1.2.1. *Important domains in NPR1*

NPR1 contains two protein–protein interaction motifs: a BTB/POZ (for Broad complex, Tramtrack, and Bric-a-brac/ Pox virus and Zinc finger) domain at the N-terminus (Bardwell and Treisman, 1994; Aravind and Koonin, 1999), ankyrin repeats in the middle of NPR1, and a transactivation domain at the C-terminus of NPR1 (Cao et al., 1997; Ryals et al., 1997; Mosavi et al., 2004).

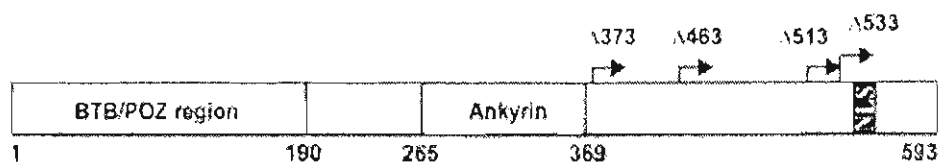


Figure 1. Scheme of NPR1 including N-terminal BTB/POZ domain, located in the first 190 residues, ankyrin repeats, residue 265 to 369, C-terminus with indication of truncated versions of CT of NPR1 and nuclear localization signal (NLS) at end of NPR1 (Rochon et al., 2006).

The BTB/POZ domain is an evolutionarily conserved protein-protein interaction domain found in developmentally regulated transcription factors. Many BTB/POZ domain-containing proteins including human promyelocytic leukemia zinc finger (PLZF), tramtrack, and BCL6, are transcriptional repressors while some including GAGA factor can counteract repression by chromatin remodelling (Ahmad et al., 1998). The three-dimensional structure of PLZF has been solved, and the study of the structure of PLZF BTB domain showed that the BTB domain is a tightly intertwined dimer with a hydrophobic interface which may be important for BTB-BTB domain interactions, and surface features which may be involved in the interactions with other proteins. The BTB/POZ domain can self associate into a homologous dimer, and also can form heterologous dimer or complexes with other non-BTB domain protein (Ahmad et al., 1998). The BTB/POZ domain PLZF was also shown to be crucial for transcriptional repression (Melnick et al., 2000). The BTB/POZ domain of NPR1 likely belongs to that of PLZF. Boyle et al (2009) showed that co-transfecting

POZ:DB and POZ:TA lead to a significant increase in luciferase activity, suggesting that the NPR1 BTB/POZ can self-associate independent of SA. This self-association of BTB/POZ domain to form a dimer was also supported by size-exclusion chromatography followed by immunoblot analysis. However, co-expression of the NPR1 BTB/POZ fused to the VP16 transactivation domain (POZ:TA) with Gal4 DB or co-expression of NPR1 BTB/POZ fused to Gal4 (POZ:DB) with VP16 TA did not activate the reporter gene.

TGA2, one of the members of the TGA transcription factor family, has been reported to be a constitutive repressor with a non-autonomous repression domain at its N-terminus, and the proteins of the TGA2-containing clade are required for basal repression of *PR-1* (Zhang et al., 2003; Rochon et al., 2006; Boyle et al., 2009). However, upon SA treatment, TGA2 associates with Arabidopsis NPR1 to form a TGA2-NPR1 enhanceosome that is essential for *PR-1* induction. In this TGA2-NPR1 complex, NPR1 functions as a transcriptional coactivator, requiring its BTB/POZ domain and oxidized two cysteines, Cys-521 and Cys-529, located in a C-terminal transactivation domain (Rochon et al., 2006). Boyle et al. demonstrated that the BTB/POZ domain of NPR1 not only interacts with, but also masks or negates the function of the repression domain of TGA2, providing a new insight into the function of the BTB/POZ domain (Boyle et al., 2009).

NPR1 encodes a protein containing ankyrin repeats. The ankyrin repeats are important for NPR1 function as they mediate interactions with TGA factors. Their mutation abolishes the interaction between NPR1 and TGA transcription factors,

therefore compromising *PR* gene expression and SAR (Cao et al., 1997; Ryals et al., 1997; Zhang et al., 1999; Després et al., 2000, 2003).

NPR1 contains an autonomous transactivation domain at its C-terminus that is found in residues 513 to 533. The domain contains two cysteines C521 and C529, which are oxidized. In SA-treated tissues, these cysteines are required for the co-activator function of NPR1 to induce *PR-1* expression and SAR (Rochon et al., 2006).

1.3. *The role of SA in SAR*

SA has been found to be an important metabolite involved in plant disease resistance, which is critical for *PR* expression and SAR. Transgenic Arabidopsis and tobacco plants that express the bacterial salicylate hydroxylase (*NahG*) gene, whose product is able to convert SA to biologically inactive catechol, fail to develop SAR and show increased susceptibility to primary infection by pathogens (Gaffney et al., 1993; Delaney et al., 1995). Apart from the influence on disease resistance, SA also influences other processes including seed germination, seedling establishment, cell growth, senescence-associated gene expression, basal thermotolerance, and fruit yield (Klessig and Malamy, 1994; Rate et al., 1999; Morris et al., 2000; Rajou et al., 2006). The effect of SA on some of these processes may be indirect because SA alters the production or signaling effectiveness of other plant hormones such as JA, ET and auxin (Vlot et al., 2009).

1.3.1. *SA metabolism*

SA in plants can be generated in chloroplast via two distinct pathways, both of which are starting from the primary metabolite chorismate. One of the pathways to synthesize SA involves a series of enzymatic reactions initially catalyzed by the enzyme Phenylalanine Ammonia Lyase (PAL). Another pathway involves two enzymatic reactions catalyzed by the Isochorismate Synthase (ICS) and Isochorismate Pyruvate Lyase (IPL). This latter pathway is the one through which a large number of SA molecules induced by pathogen is synthesized in *Arabidopsis* (Vlot et al., 2009).

Even though SA is a key molecule to activate defense genes and SAR, an excess amount of free SA may be phytotoxic. Thus, plants regulate free SA level in part by converting it into a glucosylated form, SA *O*- β -glucoside (SAG), which is the dominant form of SA in plants (Malamy et al., 1992; Lee et al., 1995; Nobuta et al., 2007). *Arabidopsis* encodes two pathogen-inducible SA glucosyltransferase (SAGT), one of which preferentially converts SA into SAG whereas another one converts SA into salicyloyl glucose ester (SGE) in much lower abundance. SAG is actively transported from the cytosol into the vacuole, where it may function as an inactive primary storage form of SA that can be converted back to SA (Lee and Raskin, 1999; Dean et al., 2004, 2005; Song, 2006). Methyl salicylate (MeSA) and its glucosylated form, methyl salicylate *O*- β -glucoside (MeSAG) have been also shown to accumulate to high levels in plants (Seskar et al., 1998; Dean et al., 2004, 2005; Park et al., 2007).

PBS3 is a member of the GH3 family of acyl-adenylate/thioester-forming enzyme that conjugates amino acid to SA to form SA-amino acid conjugation (Nobuta et al.,

2007). Amino acid conjugation of SA involving PBS3 is proposed to affect SA action because altered expression of PBS3 affects disease resistance. It was reported that PBS3 plays an important role in pathogen-induced SA metabolism and induced defense responses because its mutations impact SAG accumulation, SA-dependent transcriptional responses such as *PRI* gene expression and disease resistance (Nobuta et al., 2007).

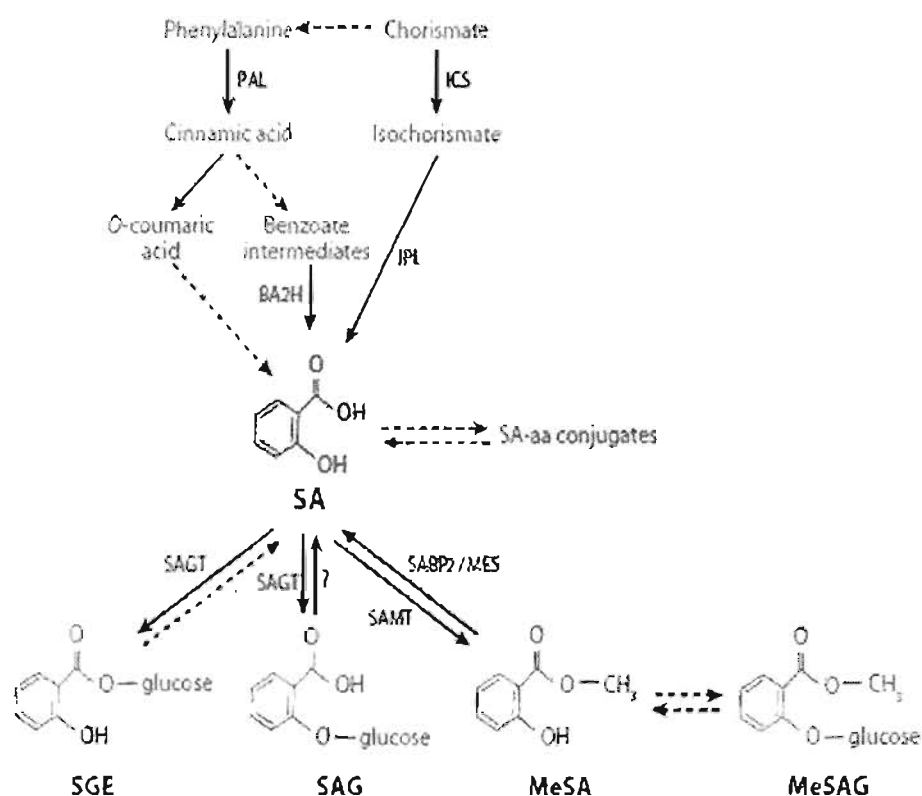


Figure 2. Simplified schematic of pathways involved in SA biosynthesis and metabolism.

Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SA, salicylic acid; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase; SGE, salicyloyl glucose ester; SAG, SA *O*-β-glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate *O*-β-glucoside.

1.3.2. *NPR1-dependent and NPR1-independent SA signalling*

The requirement of NPR1 for the induction of SAR and the activation of *PR-1* in response to SA is well documented by numerous different genetic screens (Cao *et al.*, 1994; Delaney *et al.*, 1995; Zhang *et al.*, 2003). Plants containing *npr1* mutations were first identified in screens for Arabidopsis that were not capable of activating *PR* gene expression and disease resistance in response to SAR inducer (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah, 2003). The NPR1 promoter contains a W-box to which a WRKY protein binds in vitro. The binding of an SA-inducible WRKY protein to W-box element in the promoter of NPR1 is necessary for NPR1 expression. Mutation of the W-box in the promoter of NPR1 abolishes the binding of WRKY proteins, thus compromising the ability of NPR1 to complement the *npr1* mutant (Yu *et al.*, 2001). Application of SA or its analogs stimulates the translocation of NPR1 from cytosol to nucleus, where it interacts with members of TGA transcription factors of basic/leucine zipper DNA-binding proteins, thereby activating downstream signalling. Activation of *PR* genes during SAR not only requires nuclear localization, but also depends on a functionally redundant clade of three basic leucine zipper TGA transcription factors, TGA2, TGA5 and TGA6 (Kinkema *et al.*, 2000; Després *et al.*, 2003; Durrant and Dong, 2004). TGA1, another member of TGA transcription factors, does not interact with NPR1 in yeast two-hybrid assays, but interacts with NPR1 in Arabidopsis upon SA treatment. Moreover, the DNA binding activity of the reduced form of TGA1 is stimulated by NPR1. Only in its reduced form does TGA1 interact with NPR1. TGA1 is in its reduced state after the treatment of plants with SA (Després *et al.*, 2003). In

summary, SA affects the NPR1-dependent SA signalling pathway by first activating its expression and then stimulating its translocation to the nucleus where it interacts with TGA transcription factors to induce defense gene expression and SAR.

Loss-of-function mutations in NPR1 do not confer complete loss of *PR* gene expression and disease resistance, suggesting that these responses also can be activated via an NPR1-independent pathway. The *PR-1* expression and SAR activation are also regulated in an SA-dependent but NPR1-independent manner by transcription factor WHY1 (Whirly 1) (Desveaux et al., 2004). Two *why1* mutant alleles in Arabidopsis were demonstrated to be compromised in SA-induced disease resistance, suggesting that AtWhy1 is an important downstream component of the SA-signaling pathway whose activation is independent of NPR1. Furthermore, plants with *why1* mutant alleles displayed enhanced susceptibility to infection by virulent and avirulent pathogens (Desveaux et al., 2004). Taken together, WHY1 is critical in plant defense responses in addition to NPR1.

The *ssi2* mutant, identified in a genetic screen for *suppressors of npr1*, also appears to affect the activation of defense responses by modulating an NPR1-independent defense pathway (Shah, 2001). In comparison to wild-type (SSI2 NPR1) plants, which only express *PR* genes in the absence of SA, and *npr1* mutant (SSI2 *npr1*) plants, which do not express *PR* genes either in the absence or the presence of SA, the *ssi2 npr1* double mutant and the *ssi2* NPR1 single mutant constitutively express *PR* genes and exhibit enhanced resistance to pathogens. However, disease resistance induced by *ssi2* is compromised by *NahG* but is retained in the *npr1* mutant background,

indicating that an NPR1-independent mechanism dependent of SA is involved in addition to the NPR1-dependent mechanism (Shah et al., 2001, 2003).

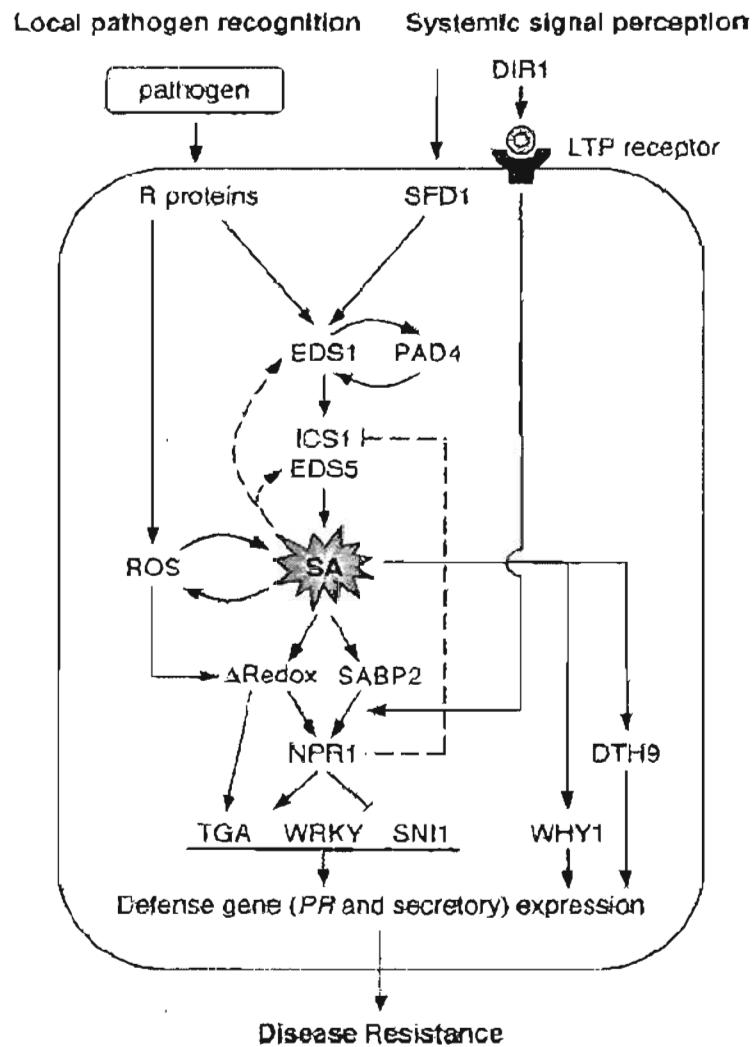


Figure 3. Sequence of events from pathogen recognition to defense gene induction deployed during disease resistance (Durrant and Dong, 2004). DIR1: defective in resistance 1, SFD1: suppressor of fatty acid desaturase deficiency1, LTP: lipid transfer proteins, EDS1: enhanced disease susceptibility 1, PAD4: phytoalexin deficient 4, ICS1: isochorismate synthase 1, EDS5: enhanced disease susceptibility 5, ROS: reactive oxygen species, SABP2: salicylic acid-binding protein 2, DTH9: detachment 9, WHY1: whirly 1, SNI1: suppressor of NPR1-1, inducible 1.

1.3.3. *SA feedback loops*

Activation of defense signalling mediated by resistance genes induces SA synthesis and downstream defense responses. In addition to expression of several R genes, application of SA also activates the expression of the *EDS1* gene, which is required for SA accumulation and activation of resistance conferred by R-gene-activated signalling pathways, suggesting a feedback regulation of these R genes and *EDS1* by SA (Feys et al., 2001; Shah, 2003). Furthermore, SA activates the expression of other SA-biosynthesis-related genes including *EDS5*, *PAD4* and *SID2* (salicylic acid-induction deficiency 2), showing multiple points where SA exerts a regulatory feedback effect important for amplifying plant defense responses (Shah, 2003).

In addition to the positive feedback loop, a negative feedback regulation involving NPR1 is also reported (Shah, 2003). In this negative feedback loop, SA accumulation and *SID2* expression are diminished in wild-type plants containing NPR1 but not in plants with an *npr1* mutant background. It is believed that uncontrolled SA synthesis may affect other signaling pathways that are inhibited by SA such as the JA-dependent signaling pathway. Therefore, both positive and negative feedback loops are important as they allow for the regulation of SA accumulation and fine-tuning of plant defense signaling.

1.3.4. *SA-binding proteins*

The investigation as to how SA exerts its effect revealed that several putative effector proteins including catalase, ascorbate peroxidase, and carbonic anhydrase bind SA

with low to moderate affinity (Kumar and Klessig, 2003). In comparison, SA-binding protein 2 (SABP2) is a protein from tobacco that displays high affinity for SA and it is involved in the SA-mediated signal transduction pathway leading to defense response to pathogen infection. The binding between SA and SABP2 is reversible and specific to SA and its SAR-inducing analogs such as benzothiadiazole (BTH).

1.3.4.1. *SABP2 and its lipase and esterase activities*

SABP2 is a member of the α/β hydrolase family that contains a catalytic triad of serine, aspartate and histidine. Crystallographic analysis showed that SA is bound in the active-site pocket of SABP2 located in the C-terminal end via hydrogen-bonding, polar and van der Waals interaction (Forouhar *et al.* 2004). The carboxylate group of SA is bound deepest in the pocket of SABP2, and one of its oxygen atoms is hydrogen-bonded to the side chain of the histidine in the catalytic triad. The phenyl ring of SA is located in a highly hydrophobic environment.

There is evidence showing that lipids play an important role in signaling during disease resistance. The Arabidopsis EDS1 and PAD4 proteins, which are putative lipases, are required to transduce the resistance signal by a specific class of R genes after pathogen recognition (Kumar and Klessig, 2003). Similar to EDS1 and PAD4, SABP2 also contains the catalytic triad and lipase signature sequence, so it has lipase activity and this lipase activity is stimulated by SA. It is speculated that SABP2 activates the plant defense system through a mechanism that postulates that the SA-stimulated lipase activity of SABP2 generates an SAR-inducing lipid that is

translocated by a lipid transfer protein to the uninfected areas of the plant. Plants with knock-down SABP2 via RNAi fail to express *PR-1* gene induced by SA after infection, thereby blocking the development of SAR. Therefore, SABP2 is required for systemic resistance to pathogen infection, and the ability of SA to regulate the lipase activity of SABP2 suggests a mechanism through which lipids are linked to the SA-dependent defense system in the plant.

In addition to the SA-stimulated lipase activity, SABP2 has esterase activity due to the presence of a serine in the catalytic triad, which is able to convert biologically inactive MeSA in systemic tissues to active SA as part of the signal transduction pathways that initiate SAR. It is also found that SA is a potent inhibitor of the SABP2's esterase activity, which acts as a competitor binding the SABP2 active site. As a result, MeSA accumulates and is then translocated to systemic tissues for perception and processing to SA by SABP2.

1.3.4.2. *Other SA-binding proteins*

Catalase, ascorbate peroxidase, and carbonic anhydrase bind to SA with much lower affinity compared to SABP2. It was shown that the increase in SA after pathogen infection inhibit the H₂O₂-scavenging activities of both catalase and ascorbate peroxidase. This inhibition results in the alteration of the cellular redox state which facilitates the relocation of NPR1 from the cytoplasm to the nucleus for activation of SA-responsive defense genes including *PR* gene expression (Mou et al., 2003; Dempsey et al., 1999). The increase in SA does not affect the activity of carbonic

anhydrase, but it appears that carbonic anhydrase is involved in plant immunity because silencing of this enzyme in plant increases the susceptibility to avirulent pathogens (Restrepo et al., 2005).

1.3.4.3. *MarR, a model for salicylate binding to protein*

Protein members from the MarR family are involved in controlling an assortment of biological functions, including resistance to multiple antibiotics, organic solvents, household disinfectants and oxidative stress agents, that are collectively known as the multiple antibiotic resistance (Mar) phenotype (Martin and Rosner, 1995; Alekshun, et.al. 2001). The emergence of multiple antibiotic-resistance bacteria is due to the presence of the *mar* regulon in *E. coli* which is inducible by the antibiotics chloramphenicol and tetracycline and aromatic weak acids including salicylate and acetylsalicylate (Martin and Rosner, 1995). The stimulated transcription of the *mar* operon initiates a cascade of events leading to multiple antibiotic resistance. However, many studies have revealed that the transcription of the operon is repressed *in vivo* by the marR-encoded protein, MarR, and derepressed by salicylate (Martin and Rosner, 1995; Alekshun, et.al. 2001).

By binding to one site of the *mar* operon where the RNA polymerase would bind, MarR could interfere with the binding of the RNA polymerase to the -35 and -10 sequences necessary for transcription, thereby inhibiting transcription of the operon. The result of electrophoretic mobility assay showed that salicylate binds to MarR

protein and weakens the interaction of MarR with *mar* DNA (Martin and Rosner, 1995).

The crystal structure of MarR shows that it binds to the *mar* operon as a dimer with each subunit containing a winged-helix DNA binding motif and with the terminal parts of each monomer contributing to a protein-protein interaction interface in the dimer. The organization of the N-terminal and C-terminal domains and of two DNA binding domains results in the formation of a wide channel through the center of the dimer (Aleksun, et.al. 2001). It is believed that salicylate binds to two sites, in each subunit of the MarR dimer, that are on either side of the proposed DNA-binding helix. Binding is via hydrogen bonds and is due to the presence of the hydroxyl and carboxyl group of SA. In each of the sites, the salicylate ring sits over a hydrophobic side chain in the pocket (Aleksun, et.al. 2001).

1.4. Metalloproteins

Approximately one-third of all known proteins contains metal ion cofactors and are known as metalloproteins. The majority of them function as essential metalloenzymes. Since protein atoms such as oxygen, nitrogen and sulfur coordinate with metals, for example, zinc, copper, and iron through covalent bonds in metalloproteins, amino acids containing these atoms have been shown to be critical for the function of metalloproteins (Bartnikas and Gitlin, 2001; Kennedy et al., 2001).

To date, metalloproteins have been well-characterized into four major categories, which are transport proteins, electron transport proteins, signal transduction proteins

and metalloenzymes. (1) Transport proteins are responsible for molecular transport such as oxygen transport. The hemoglobin, an iron-containing metalloprotein, is a typical example of this class that carries oxygen in the bloodstream; (2) Since many metals have stable oxidation states that are energetically accessible at physiological conditions, they are ideal for electron transport in processes involving redox reactions. One example of electron transport proteins are the cytochromes containing iron; (3) Many transcription factors may also be found to be metalloproteins. The well-known example is the zinc finger protein domain of transcription factor containing a zinc ion in its centre by which cysteines and histidines are coordinated; (4) Metalloenzymes are proteins that function as enzymes and contain metals to which proteins are tightly bound. In metalloenzymes, the metals are usually located in a pocket into which small substrates fit. These metalloenzymes include superoxide dismutase containing copper or zinc, alcohol dehydrogenase containing zinc and so on.

One previous work demonstrated a novel mechanism to make a metalloprotein using the Cu/Zn superoxide dismutase (SOD1) as a model, revealing that the delivery and insertion of copper to SOD1 within a cell is facilitated by a metallochaperone, the copper chaperone for SOD1 (CCS). The process involves a direct interaction of the chaperone with the metal-binding site of the target protein SOD1 via a disulfide bond (Bartnikas and Gitlin, 2001). Three domains were found in CCS. One is MXCXXC (where X is any amino acid residue), a copper binding motif at the NT of CCS. The second domain is highly similar to that of SOD1, but lacks the critical amino acids required for catalysis. The third domain contains a highly conserved CXC motif that

is critical for copper delivery, but is not needed for protein-protein interactions (Bartnikas and Gitlin, 2001). Because of this structure, CCS can be used for delivery and insertion of copper to SOD1.

According to one previous study, based on the structure of SA, it is capable of coordinating with metals such as copper through its carboxylate groups (Abuhijleh and wood, 2001). Since it was also known that SA is involved in the reduction of NPR1 oligomer, it is possible that NPR1 may serve as a SA receptor. Taken together, one can speculate that the binding of SA to NPR1 could be metal-dependent. In order to confirm this hypothesis, one of the experiments to do can be the examination of metal-binding capacity of NPR1 to test whether NPR1 is a metal-binding protein in addition to the investigation of direct binding of SA to NPR1.

Chapter 2. Materials and methods

2.1. Chemicals

All consumables were purchased from Sigma (St. Louis, MO) unless otherwise stated.

Media for growing *E. coli*

2xYT media

per liter

- Tryptone 16g
- Yeast extract 10g
- NaCl 5g

LB media (Luria-Bertani Medium)

per liter

- Tryptone 10g
- Yeast extract 5g
- NaCl 10g

-Both of the 2YT and LB media were made according to the recipes listed above by mixing all the chemicals and then dissolving by adding water.

-They must be sterilized by autoclaving before use.

Biological buffers

1.Ni-NTA column (1mL):

(A) Stripping buffer (100ml): 0.276 NaH₂PO₄ (20mM), 2.922g NaCl (500mM), 1.861g EDTA (50mM), pH 7.4

(B) Binding buffer (100ml): 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 2ml 2M imidazole (40mM), pH7.4

(C) Elution buffer (100ml): 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 6.81g imidazole (1M), pH7.4

-Before each use, the Ni-NTA column has to be stripped with stripping buffer containing EDTA to get rid of any proteins and metals that are not eluted completely out of the column by the elution buffer from the previous experiment. It is then recharged with Ni²⁺. In addition to the binding and elution buffers listed above, binding and elution buffers containing 100mM EDTA (Bioshop) are also used to test the presence of metal-binding protein in my experiments.

2.Strep-Tactin column (1mL):

(A) Regeneration buffer (100ml): 0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), 0.024g HABA (1mM), pH8.0

(B) Binding buffer (100ml): 0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), pH8.0

(C) Elution buffer (100ml): 0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), 0.054g desthiobiotin (2.5mM), pH8.0

-Before each use, the Strep-Tactin column always has to be washed with regeneration buffer containing HABA to get rid of all the biotin from the elution buffer remaining on the matrix of column. In addition to the elution buffer as listed above for the Strep-Tactin column, the elution buffer containing 1mM SA is also used for my experiment.

2.2. Methods

2.2.1. Bacterial strain, general cloning vector and transformation of plasmids

E. coli BL21 strain (DE3) was transformed with a vector containing the gene of various deletions of NPR1. The genes from the N-terminus of NPR1 were amplified by PCR and cloned in-frame in pET41a (Novagen) to create N-terminal fusions (with an N-terminal Strep-tag) while the genes from the C-terminus of NPR1 were fused to an HA-tag. Plasmid purification was performed using MiniPrep Kits (Qiagen). All constructs used were verified by sequencing.

2.2.2. Expression of proteins

A five ml culture in 2xYT media containing 50mg/ml kanamycin was inoculated, and grown overnight on a rotary shaker at 250rpm at 37 °C. All 5 ml of this saturated culture was inoculated in 1L of LB (Luria-Bertani) media containing 50mg/ml kanamycin, induced with 0.5M IPTG (Isopropyl- β -D-thiogalactopyranoside; BioShop) when the OD600 of this culture reaches 0.4-0.6 and grown for an additional 2hr with rotary shaker at 250rpm at 37 °C. After 2hr, the cells were harvested by centrifugation

at 8000rpm for 10min (Sorvall-RC-5C-Plus; Mandel Scientific Company). The cells were washed with MQ H₂O and centrifuged at 15000 rpm at 4 °C for 5min. The supernatant was discarded and the pellet was stored at -20 °C until needed.

2.2.3. Purification of proteins by pull-down assays using affinity chromatography and detection of protein using western blot

The bacterial pellets were resuspended in binding buffer (0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), pH8.0 for Strep-Tactin column/ 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 2ml 2M imidazole (40mM), pH7.4 for Ni-NTA column), sonicated (Sonic Dismembrator Model 100; Fisher Scientific) on ice, and subsequently centrifuged at 15000 rpm at 4 °C for 20min to separate protein from cell debris. The supernatant was filter sterilized (0.45µm) and was then applied to the Strep-Tactin column/Ni-NTA column pre-equilibrated in 5ml binding buffer.

The protein was purified using the ÄKTA Explorer FPLC (GE Healthcare Bio-Sciences) operated with the UNICORN Version 3.00.10 software with a Strep-Tactin 1mL cartridge (Qiagen)/ Ni-NTA 1mL column (Healthcare) according to the manufacturer's instructions. The filtered protein crudes were loaded onto the column after a 5mL equilibration of the column with binding buffer. The column was then washed with 10mL binding buffer followed by 5mL elution buffer (0.69g NaH₂PO₄ (50mM), 1.75g NaCl (300mM), 0.054g desthiobiotin (2.5mM), pH8.0 for Strep-Tactin column/ 5ml 1M HEPES (50mM), 3ml 5M NaCl (150mM), 6.81g imidazole (1M), pH7.4 for Ni-NTA column) which was collected in 12 × 500µL

fractions in preparation for analysis by SDS-PAGE. The concentrations of protein in the crude, flow through and wash fractions were determined using the Bio-Rad protein assay reagent (Bio-rad). The collected 10 fractions (0.5 ml/ fraction) were acetone precipitated. The acetone precipitated elutions and thirty μg (or micro liter) of crude, flow through, wash were subjected to SDS-PAGE (14% gel). An immunoblot analysis with an anti-Strep-tag antibody (Qiagen) at a 1:2000 dilution and a monoclonal anti-HA-tag antibody (Santa Cruz Biotechnology) at a 1:1000 dilution were used to detect the presence of the BTB/POZ domain fused with a Strep-tag and the proteins generated from the CT of NPR1 with an HA-tag, respectively.

2.2.4. Plant in vivo transcription assays and two-hybrid assays

All procedures for plant in vivo transcription assays and plant two-hybrid assays were previously described (Després et al., 2003). All constructs were created by PCR as previously described (Rochon et al., 2006). *Arabidopsis thaliana* (ecotype Columbia) leaves were harvested from 4-week-old plants grown at 21 °C (day) and 18 °C (night) with a 10-h photoperiod and transferred to Petri dishes containing MS salts and micronutrients supplemented with B5 vitamins, 1% sucrose, and 0.8% agar at pH 5.8. When required, filter-sterilized salicylic acid was added to the medium at a final concentration of 1mM. Coating of the gold particles and general procedures and preparation of the biolistic experiments (bombadment) were as described according to the manufacturer's instructions (Bio-Rad). One microgram of the reporter plasmid (firefly luciferase), 0.1 μg of internal standard plasmid (renilla), and 1 μg of each

effector plasmid were mixed together and the mixture was used to coat beads. Assays were performed using the Dual-Luciferase Reporter Assay system (Promega) following the manufacturer's instructions. Luminescence was measured on a Berthold Lumat LB9507 luminometer (Bad Wildbad), and the data obtained represent the value of the reporter gene (firefly luciferase) divided by the value of the internal standard (renilla) and expressed as relative luciferase units. Each bar in each graph represents five bombardments repeated five times (25 values per point).

2.2.5. Analysis of protein by gel filtration

All the procedures for the gel filtration were described previously (Boyle et al., 2009). Strep-tagged purified proteins were diluted to the required concentrations using S300 running buffer (50 mM HEPES and 250 mM NaCl, pH 7.4) prior to gel filtration analysis on the Sephacryl S300 HR packed in 50-cm-long HR 16 column (GE Health) and equilibrated with S300 running buffer. Elutions were performed in the same buffer containing different chemicals (1mM SA/1mM catechol/20mM DTT) at a flow rate of 0.8 mL/min. Each fraction contains 0.5 mL elution.

Chapter 3. Results

3.1. The BTB/POZ domain at the NT of NPR1 functions as an inhibitory domain to repress the transactivation domain at the CT of NPR1

According to a previous study, NPR1 contains an autonomous transactivation domain in its last 80 amino acids and requires the stretch of residues located between positions 513 and 533, which contains two cysteines (Cys-521 and Cys-529) as shown in Figure 4A and 4B (Rochon et al., 2006). An N-terminal deletion of NPR1 at residue 513 was engineered at the beginning of a stretch of negatively charged and hydrophobic residues, a signature of transactivation domains. A second one, starting at residue 533 starts right before the nuclear localization signal. This deletion effectively removes the two Cys residues (Rochon et al., 2006). Cys-521 and Cys-529 are predominantly oxidized, regardless of whether SA treatment is present or not, and they are required for transactivation only upon SA treatment. To identify whether there is an inhibitory domain in NPR1, the full-length NPR1 and several N-terminal deletions of NPR1 are fused to Gal4 DB, and were assayed using an *in vivo* plant transcription assay (Figure 4C). The baseline level of transcription was determined by transfecting leaves with Gal4 DB alone with a reporter construct consisting of a firefly luciferase gene under the control of five copies of the Gal4 upstream activating sequences (UAS) fused to a minimal promoter (Rochon et al., 2006). The result in Figure 4C showed that transfection with the full-length NPR1:DB led to reporter gene repression below the baseline level without SA, indicating that the full-length NPR1

did not transactivate without SA. However, transfection with two N-terminal deletion mutants of NPR1, $\Delta 513$:DB and $\Delta 533$:DB, exhibited reporter gene activation beyond the baseline level, suggesting that the two proteins did transactivate without SA. This result is consistent with a previous result showing that $\Delta 513$ at the CT of NPR1 is a transactivation domain (Rochon et al., 2006). As observed in Figure 4C, $\Delta 513$:DB resulted in a much more significant increase in reporter gene expression compared to that of $\Delta 533$:DB, suggesting that the two Cys at the CT of NPR1 could play an important role in transactivating. Taken together, these results suggest that there is an inhibitory domain in NPR1. Since previous results showed that the POZ domain could inhibit the DNA binding of the zinc finger protein ZID (zinc finger protein with interaction domain) (Bardwell and Treisman, 1994), we speculated that the POZ domain of NPR1 may have a similar role as an inhibitory domain, but this time to inhibit the transactivation domain at the CT of NPR1 via an interaction between BTB/POZ domain and CT of NPR1. Thus, we tested whether the BTB/POZ domain can interact with $\Delta 513$ in vivo by using plant two-hybrid assay and in vitro by using pull-down assays.

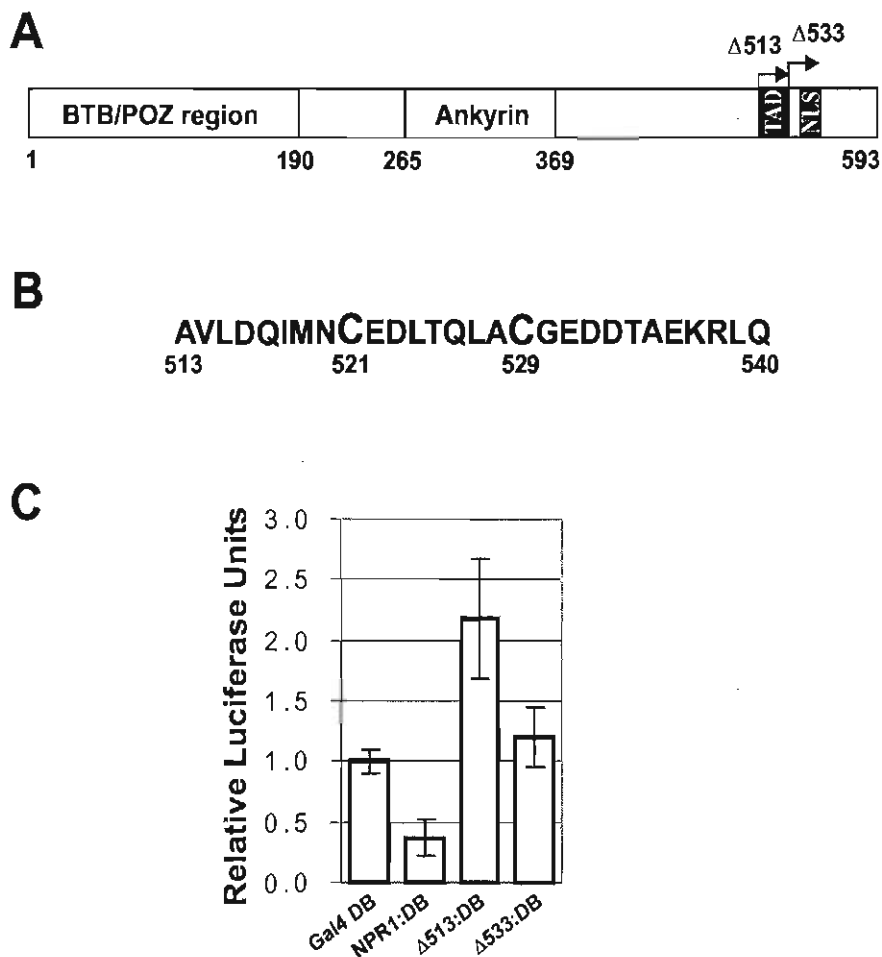


Figure 4. BTB/POZ domain at NT of NPR1 inhibits the transactivation domain at the CT of NPR1.

(A) Scheme of NPR1 showing the BTB/POZ domain, the ankyrin repeats, the C-terminal transactivation domain (TAD), the NT deletions of NPR1 analyzed in (C), and the nuclear localization signal (NLS). Numbers preceded by Δ indicate the starting amino acid for that particular deletion mutant.

(B) Sequence of amino acids located between positions 513 and 540 of NPR1. Two Cys are observed in position 521 and 529.

(C) Histograms illustrating the transcriptional activity of NPR1 and NPR1 deletions described in (A) tethered to DNA through Gal4 DB.

3.2. The N-terminal BTB/POZ domain of NPR1 interacts with the C-terminal domain of NPR1 in vivo and in vitro

Because of a previous study showing that BTB/POZ domain of the ZID protein is inhibitory (Bardwell and Treisman, 1994) and our results from Figure 1 revealing that there is an inhibitory domain present in the Arabidopsis NPR1 (Rochon et al., 2006), we tested whether the BTB/POZ region can interact with $\Delta 513$ in vivo and in vitro. To accomplish this, the BTB/POZ domain of NPR1 was fused to the Gal4 DB (POZ:DB) and $\Delta 513$ was fused to the VP16 TA ($\Delta 513$:TA). Both constructs were assayed using plant two-hybrid assays in the absence and presence of SA treatment. In the plant two-hybrid system, reporter gene activation is based on the reconstitution of a functional transcription factor, which occurs when a protein fused to the DNA-binding domain of the yeast transcription factor Gal4 interacts with a fusion protein linked to the VP16 transcription activation domain. Transfection of the reporter gene and the internal standard along with Gal4 DB served to determine the baseline level of the system (Boyle et al., 2009). Coexpressing the $\Delta 513$ fused to VP16 TA ($\Delta 513$:TA) with Gal4 DB did not lead to reporter gene expression beyond baseline. The same result was shown when coexpressing the BTB/POZ fused to Gal4 DB (POZ:DB) along with VP16TA in Figure 5A. It was also observed that in the absence of SA (white bars), expressing NPR1 BTB/POZ fused to Gal4 DB (POZ:DB) along with $\Delta 513$ fused to VP16 TA ($\Delta 513$:TA) led to a significant increase in normalized luciferase activity, suggesting that POZ:DB interacts with $\Delta 513$:TA

(Figure 5A). However, coexpressing the POZ:DB and $\Delta 513$:TA upon treatment with SA (grey bars), did not activate the reporter gene, indicating that the interaction of POZ:DB with $\Delta 513$:TA was abolished. This result suggested that SA may function as a competitor interfering with the interaction between the BTB/POZ domain and $\Delta 513$. The mode of action of SA as a competitor is not well understood and could probably occur through a binding similar to what is observed with SABP2 or other SA-binding proteins as reported previously (Kumar and Klessig, 2003). Furthermore, it was found that cotransfecting POZ:DB and POZ:TA led to a significant increase in expression beyond baseline, indicating that BTB/POZ domain can self-associate regardless of SA treatment as shown in Figure 5A, which is consistent with previous observations showing that BTB/POZ is capable of interacting with itself.

In addition to examining the interaction of the BTB/POZ domain with $\Delta 513$ by using plant two-hybrid assays *in vivo*, this interaction was further assessed by using pull-down assays *in vitro*. The basis of the pull-down assay is that a purified protein with a tag specific for an immobilized ligand in a column will capture and “pull-down” its interacting partner. BTB/POZ domain at NT of NPR1 was fused to a Strep-tag which binds with high selectivity to Strep-Tactin, an engineered streptavidin. The binding affinity of the Strep-tag to Strep-Tactin is nearly 100 times higher than to streptavidin (Qiagen). The elution of purified recombinant protein was performed by the addition of biotin or desthiobiotin, which is a natural ligand of streptavidin. In our experiment, the BTB/POZ domain of NPR1 was applied to the Strep-Tactin column under physiological conditions. Subsequently, $\Delta 513$ fused to a HA-tag was incubated

with this column to investigate whether it could interact with the BTB/POZ domain. In our pull-down assays, we used two-step elutions in which proteins were first eluted with 1mM SA elution, to evaluate whether SA has an effect on the NPR1-BTB/POZ interaction, followed by a biotin elution, to ensure that all POZ-Strep molecules elute from the Strep-Tactin column. As observed in Figure 5B, the concentration of the BTB/POZ domain input is much lower than that of the $\Delta 513$ input. This was due to the low level expression of the BTB/POZ domain of NPR1. The immunoblot analysis with an anti-Strep antibody was used to detect the presence of the BTB/POZ domain and to confirm that this domain binds to and elutes from the Strep-Tactin column using biotin elution. As expected, BTB/POZ domain is eluted with biotin but not with SA (first panel of Figure 5B). On the other hand, the presence of $\Delta 513$ was detected using an anti-HA antibody after SA elution, indicating that $\Delta 513$ interacts with BTB/POZ, but that the addition of SA interferes with this interaction (second panel of Figure 5B). This result obtained in vitro is consistent with the in vivo result revealing that BTB/POZ domain interacts with $\Delta 513$, but this interaction is abolished in the presence of SA. Furthermore, $\Delta 513$ could not be detected after a biotin elution following the SA elution, suggesting that all $\Delta 513$ molecules were eluted with SA (second panel of Figure 5B).

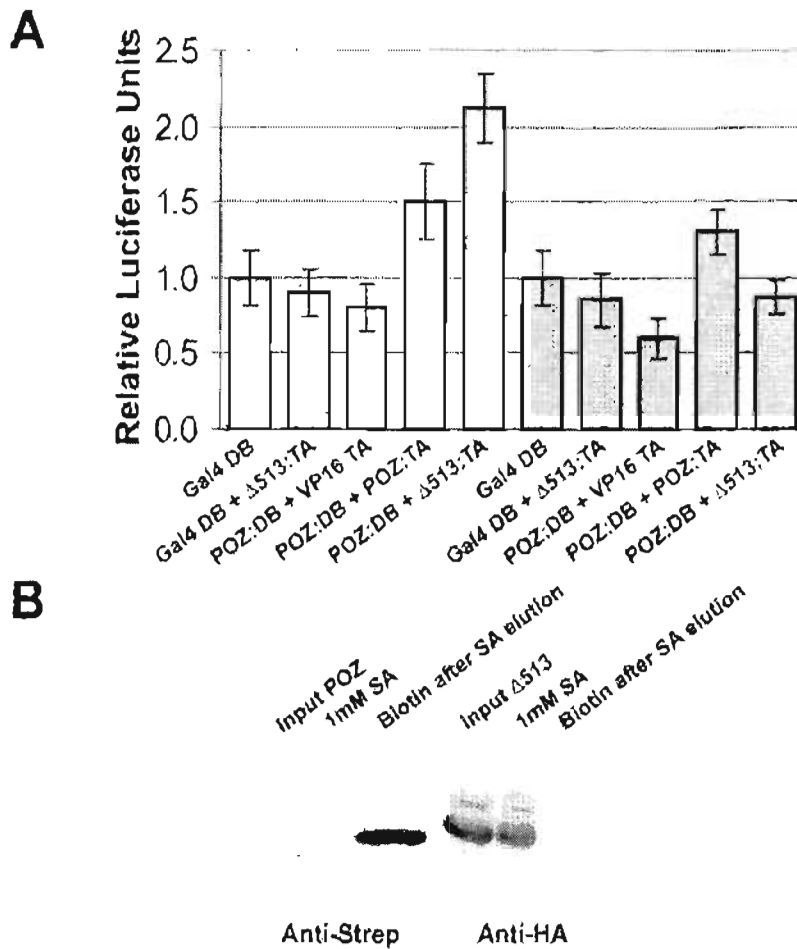


Figure 5. The BTB/POZ domain at the NT interacts with Δ513 at the CT of NPR1.

(A) Bar graph illustrating the interaction between BTB/POZ domain at the NT and Δ513 at the CT of NPR1. The BTB/POZ region is tethered to DNA through Gal4 DB (POZ:DB) or to the VP16 transactivation domain (POZ:TA). Δ513 is fused to the VP16 transactivation domain (Δ513:TA). White bars indicate the absence of SA whereas the grey bars mean the treatment of SA.

(B) Immunoblot analysis of the interaction of the BTB/POZ domain with Δ513 after pull-down assay. The BTB/POZ domain and Δ513 are revealed using an anti-StrepTag and anti-HA antibodies, respectively. In the pull-down assay, the proteins are eluted off the 1mL Strep-Tactin column with 1mM SA first and then with 2.5mM biotin elution. Lane 2 and 3 in both panels indicate the eluent from SA and biotin elutions, respectively.

3.3. NPR1 is a receptor for SA

Since it appears that SA affects the interaction between the BTB/POZ domain and $\Delta 513$ (Figure 5), we hypothesized that Arabidopsis NPR1 may function as an SA receptor. To test this hypothesis, gel filtration experiment on Sephacryl S300 was performed. It has been demonstrated that it is difficult to produce NPR1 in a soluble form in the amounts required for gel filtration (Boyle et al., 2009). To circumvent the problem, Rochon and colleagues opted to express a variant of NPR1 mutated in the core of the BTB/POZ domain through Ala substitutions (Rochon et al., 2006). Since this mutation affects BTB/POZ domain dimerization, it is not a perfect solution, but it yielded a sufficiently high concentration of soluble protein to perform the gel filtration experiment (Melnick et al., 2000; Boyle et al., 2009). In spite of this imperfection this mutant was used for the development of gel filtration experiments aimed at investigating whether SA has any effect on full-length of NPR1.

As shown in Figure 6A, when analyzed on its own, NPR1 eluted exclusively in the void volume (fraction 55). When treated with 1mM of SA, NPR1 could still be found in the void volume (fraction 55) but also in fractions 90 to 95, indicating that NPR1 was only partially redistributed. This observation has led to a conclusion that SA affects the conformation of NPR1. Previous studies reported that SA induces SAR and *PR* gene expression. However, catechol, a degraded form of SA produced by the enzyme *NahG* fails to develop SAR and express *PR* genes because of the reduction of a carboxylate group to a hydroxyl group. The effect of catechol on full-length of NPR1 was then examined. As observed in Figure 6A, when NPR1 was treated with

catechol, NPR1 was only found in the void volume but not in other fractions, suggesting that the degraded form of SA has no effect on NPR1 conformation. Therefore the experiment with catechol serves as a negative control. Next, gel filtration after treatment of NPR1 with dithiothreitol (DTT), a reducing agent, was performed. Results revealed that DTT does not change the structure of NPR1. Together, these results suggested that NPR1 may be an SA receptor, but future work is required to test whether the binding of SA to NPR1 is direct or indirect.

The stoichiometry of NPR1 in 1 mM SA was also evaluated by using gel filtration on Sephacryl S300. To accomplish this, a Sephacryl S300 calibration curve was established and S300 gel filtration analyses, aimed at determining the elution fractions and corresponding volumes, were performed. As observed in the immunoblot analysis of the NPR1 + SA panel in Figure 6A, besides the detection of NPR1 in the void volume, the highest amount of NPR1 found in the included volume was in fractions 90 and 95. According to Table 2 (predicted fractions as a function of NPR1 stoichiometry), one can see that only the predicted fraction 94 (NPR1 dimer) is located between fraction 90 and 95 of Figure 6 (the fractions which contain the highest amount of NPR1). This suggests that NPR1 exists as a dimer after SA treatment in addition to its detection as an oligomer in void volume.

Since an NPR1 variant Ala-substituted in the BTB/POZ domain was used for gel filtration, we sought to determine whether this mutation affected the interaction with the CT using the plant two-hybrid system. Similarly, transfection of reporter gene and the internal standard along with Gal4 DB served to determine the baseline of the

system. In this assay, BTB/POZ domain with its core substituted with Ala was fused to Gal4 DB (A-SubPOZ:DB), whereas the N-terminal deletion $\Delta 513$ was fused to VP16 TA ($\Delta 513$:TA). A-SubPOZ:DB alone with VP16 TA did not activate the reporter gene in the absence (white bars) and presence of SA (gray bars) (Figure 6B). As observed in Figure 6B, coexpressing A-SubPOZ:DB and $\Delta 513$:TA in the absence of SA did result in a significant increase in normalized luciferase activity, which would suggest that A-SubPOZ interacts with $\Delta 513$ without SA treatment. This interaction was abolished upon SA treatment since coexpression of A-SubPOZ:DB and $\Delta 513$:TA did not activate reporter gene expression beyond baseline. Comparing the result using the A-SubPOZ (Figure 6B) to those using the wild-type BTB/POZ (Figure 5A) indicated that the A-SubPOZ mutant behaves like the normal POZ domain in vivo.

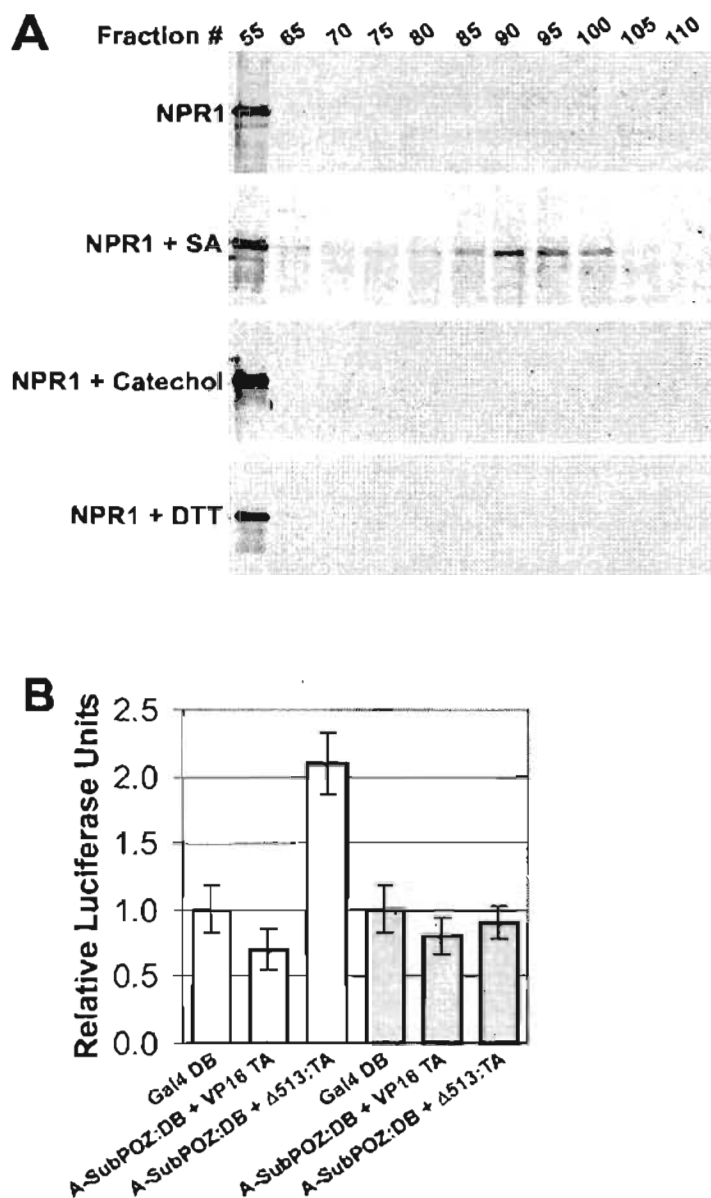


Figure 6. NPR1 is an SA receptor.

(A) Immunoblot analysis of pooled fractions from a Sephacryl S300 chromatography using an anti-Strep antibody. Fraction 55 in first lane indicates the fraction collected from the void volume. The NPR1 used in this experiment has alanine mutations in the core region of the BTB/POZ. Catechol is the product resulting from the conversion of SA by *NahG*. DTT is a reducing agent.

(B) Histograms illustrating the effect of SA on NPR1 containing alanine mutations in the core region of the BTB/POZ domain (A-SubPOZ). The BTB/POZ domain with Ala-substitution is tethered to DNA through Gal4 DB (A-SubPOZ:DB) whereas the Δ513 is fused to the VP16 transactivation domain (Δ513:TA). White bars indicate the absence of SA while gray bars mean the presence of SA treatment.

Table 1. Operational Parameters of the S300 Gel Filtration Column.

V_t (Total bed volume of the column) = 100.5 ml

V_o (Void volume of the column evaluated with Blue Dextran 2000) = 38 ml

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

MW Standards	kDa	Log MW	Ve (elution volume in ml)	Kav
Ferritin	440	2.643452676	48.27	0.16432
Catalase	232	2.365487985	53.62	0.24992
Aldolase	158	2.198657087	55.234	0.275744
Bovine Serum Albumin	67	1.826074803	59.942	0.351072
Ovalbumin	43	1.633468456	65.774	0.444384

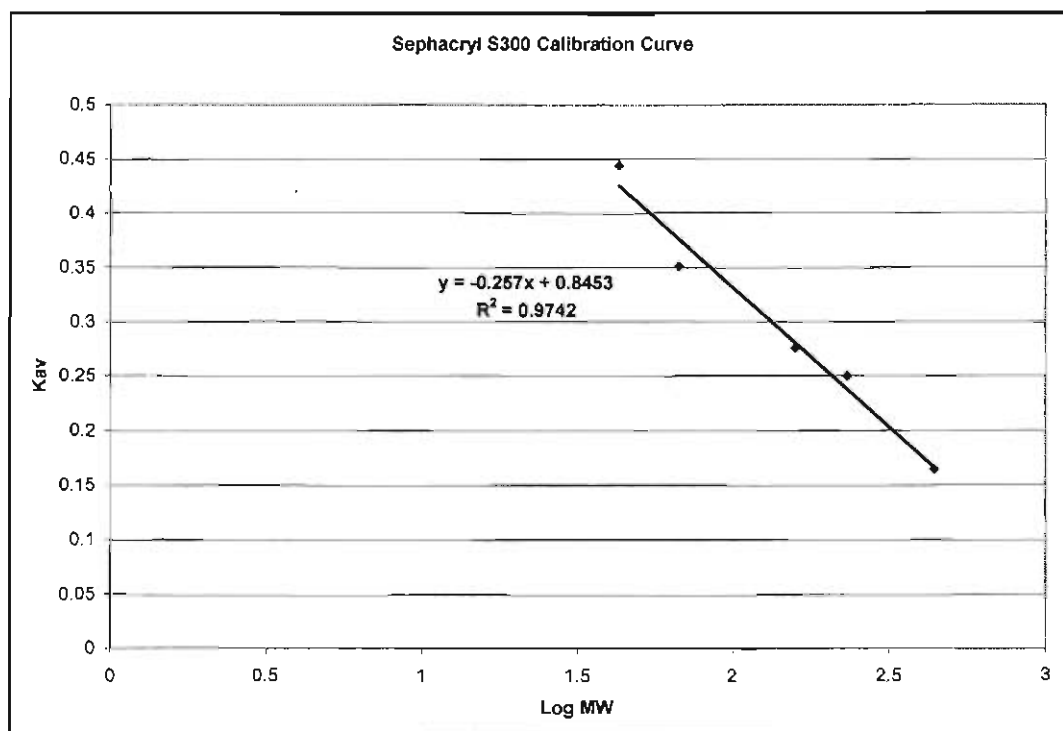


Figure 7. Calibration curve obtained for the S300 gel filtration column. Five protein standards (Ferritin, catalase, aldolase, BSA, and ovalbumin) are used to establish a Sephacryl S300 standard curve in gel filtration.

Fraction #	Elution volume (ml)	
	From	To
50	34.567	35.067
55	37.117	37.617
60	39.601	40.101
65	42.118	42.618
70	44.635	45.135
75	47.119	47.619
80	49.636	50.136
85	52.16	52.66
90	54.644	55.144
95	57.127	57.627
100	59.618	60.118
105	62.135	62.635
110	64.659	65.159
115	67.143	67.643

Figure 8. Elution fractions and corresponding volumes for the S300 gel filtration analyses. Each fraction contains approximately 0.5 ml.

Table 2: Predicted and observed elution volumes establishing the NPR1 stoichiometry in 1 mM SA.

Data relating to Figure 7 (S300 Calibration curve).

Anticipated Species	MW (kDa)	LogMW	Kav (Predicted)	Predicted Ve (mL)	Predicted Fraction number
NPR1 monomer	66	1.819543936	0.377677209	61.60482554	103-4
NPR1 dimer	132	2.120573931	0.3003125	56.76953123	94
NPR1 trimer	198	2.29666519	0.255057046	53.94106538	88
NPR1 tetramer	264	2.421603927	0.222947791	51.93423692	84

Note: the calculation of data in this Table is based on the S300 calibration curve in Fig.7 and elution fractions and corresponding volumes in Fig.8.

3.4. NPR1 acts as a metal-binding protein

The metal-binding capacity of NPR1 was tested using Ni-NTA (nickel-nitrilotriacetic acid). Based on the principle of Ni-NTA, NTA coordinates Ni^{2+} through its carboxylate groups serving as a tetradentate ligand. Based on the valency of Ni^{2+} , the column offers the possibility of binding a bidentate ligand. Usually, this column is used to purify proteins with a His-tag, which are then eluted with an elution buffer containing high concentration of imidazole. This molecule functions as a competitor of the His-tag because of its structure similar to histidine. The BTB/POZ domain of NPR1 fused to a Strep-tag at the NT was first tested for binding to Ni-NTA to see whether it could interact with a metal. The result showed that it did not bind to Ni-NTA since no BTB/POZ domain was eluted with a high concentration of imidazole (data not shown).

The metal-binding capacity of CT of NPR1 was then investigated using Ni-NTA by pull-down assays. The N-terminal deletion $\Delta 513$ fused with a HA-tag was chosen and could be detected using an anti-HA antibody. The reason for using $\Delta 513$ is that it contains two Cys residues. Cysteines have been shown to be capable of metal binding, which is observed in proteins containing zinc fingers. As shown on top panel of Figure 9, $\Delta 513\text{HA}$ was collected from both flow-through and eluted fractions (starting from fraction 4 to fraction 10), which suggests that $\Delta 513$ binds to Ni^{2+} or that it could function as a metal-binding domain. To further confirm this hypothesis, EDTA, a chelating agent, was used in the preparation of the $\Delta 513\text{HA}$ and to strip the Ni-NTA of associated nickel. The result revealed that all $\Delta 513$ directly passed through the

column and collected in the flow-through, indicating that the binding of $\Delta 513$ to column was prevented by the metal chelator EDTA. This result confirmed the previous observation that $\Delta 513$ is a novel metal-binding domain. As demonstrated previously, two Cys residues are present in $\Delta 513$ and are located in position 521 and 529 (Figure 4B). In order to examine whether the two Cys residues are important for metal binding, $\Delta 513$ HA with two Cys residues mutated ($\Delta 513$ C521S/C529S) was used. It was found that mutation of these two Cys in $\Delta 513$ HA prevented the binding of $\Delta 513$ HA to Ni^{2+} because no protein was detected in the eluted fractions as shown in Figure 9 ($\Delta 513$ C521S/C529S). Similarly, the result of pull-down assay using $\Delta 533$ HA which lacks the two Cys residues through deletion, showed all of the $\Delta 533$ HA was collected in the flow-through. Together, these results suggested that these two Cys residues in position 521 and 529 are important for $\Delta 513$ HA binding to Ni^{2+} , which is consistent with previous finding showing that Cys residues are important for metal binding in zinc finger proteins. Since NPR1 is a novel metal-binding protein, similar experiments conducted with other metals, for example Zn^{2+} and Cu^{2+} , may also be performed before drawing any conclusions about the specificity for a given metal.

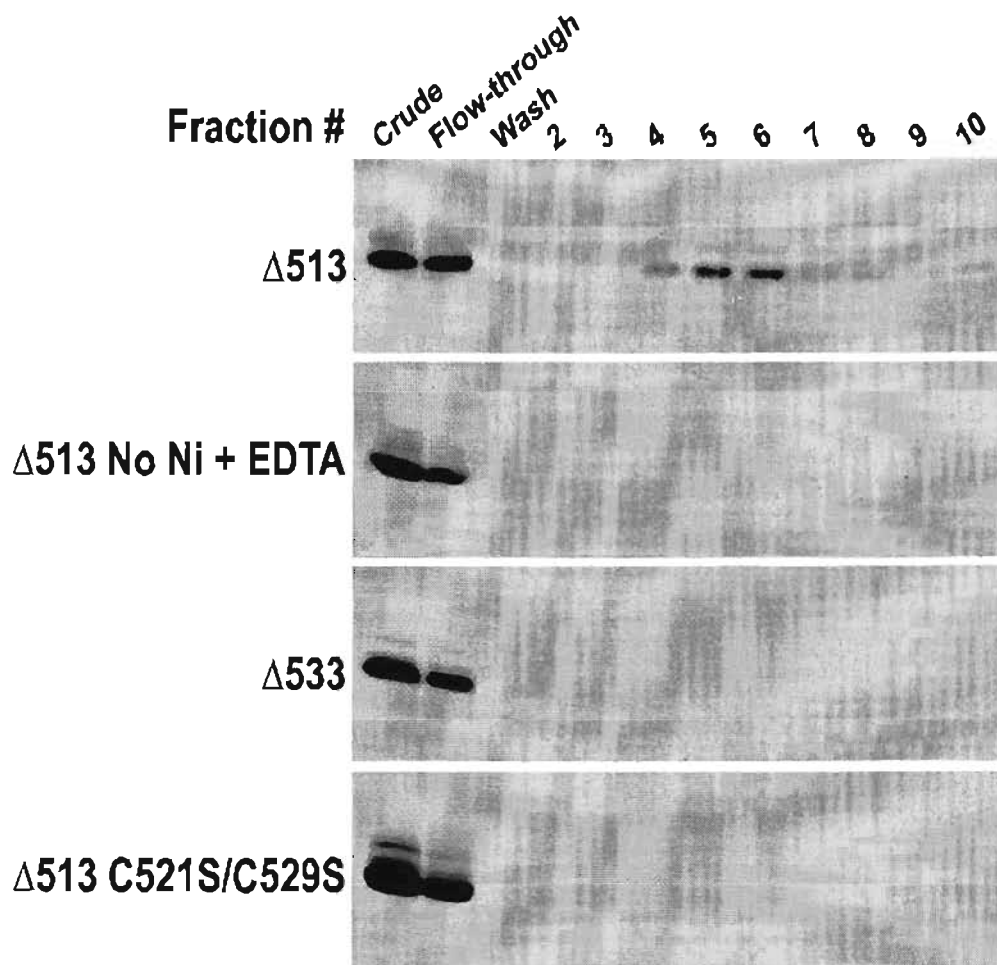


Figure 9. Immunoblot analysis of the $\Delta 513$ HA N-terminal deletion of NPR1 binding to Ni-NTA, resolved by 14% SDS-PAGE and detected using an anti-HA antibody. Thirty micrograms of crude, flow-through, and wash fractions are loaded onto the gel. EDTA is a chelating agent. C521S/C529S indicates mutations simultaneously in both Cys-521 and Cys-529. Binding buffer contains 40mM imidazole whereas elution buffer contains 1M imidazole. Number 2-10 indicate fraction 2-10 containing the eluent collected from the Ni-NTA. Each fraction contains approximate 0.5mL.

Chapter 4. Discussion

Although Arabidopsis NPR1 is well known to play an important role in regulating *PR* gene expression and SAR induction after SA treatment (Després et al., 2003; Kumar and Klessig, 2003; Verk et al., 2008), the mode of action of SA in regulating *PR* genes remains unclear. Previous studies have shown that *PR-1* gene activation requires the association of TGA2 with NPR1 which occurs upon SA treatment. This complex forms an enhanceosome that requires the core of the NPR1 BTB/POZ domain and the oxidation of Cys-521 and Cys-529 located in the transactivation domain at the CT of NPR1 (Rochon et al., 2006). However, the question of how SA is involved in the *PR-1* gene expression is still unknown. The results in our studies have shed some light on the mode of action of SA. Our results showed that the BTB/POZ domain at the NT of NPR1 interacts with $\Delta 513$ at the CT of NPR1, in vivo and in vitro, in the absence of SA treatment only (Figure 5). The data suggest that the BTB/POZ domain masks the autonomous transactivation domain containing two oxidized Cys at the CT of NPR1. Previous studies have reported a similar inhibitory function as that of the BTB/POZ domain of NPR1. For example, in the case of a zinc finger protein containing a BTB/POZ, the domain has been shown to inhibit the DNA binding activity of the zinc finger region (Bardwell and Treisman, 1994). In addition, the NPR1 BTB/POZ domain has been shown to be inhibitory to the N-terminal repression domain of TGA2 as it interacts with it to mask its function. The BTB/POZ domain also precludes the oligomeric form of TGA2 from binding to its cognate DNA sequence (Boyle et al., 2009). The in vivo and in vitro assays showed that in the

presence of SA, the interaction of BTB/POZ domain with $\Delta 513$ is abolished, suggesting that SA competitively binds with $\Delta 513$ (Figure 5 and 6). Since SA breaks the interaction between the BTB/POZ domain and $\Delta 513$, the transactivation domain at the CT of NPR1 becomes exposed, thereby leading to the formation of an enhanceosome comprised of NPR1 and TGA2, which finally results in *PR-1* gene activation (Rochon et al., 2006; Boyle et al., 2009). From the in vivo plant two-hybrid assays, it is difficult to conclude whether the binding of SA to NPR1 is direct or indirect, or whether any post-translational modifications such as phosphorylation is involved in disrupting the interaction between the BTB/POZ and the CT of NPR1. However, the in vitro pull-down assays indicated that the binding of SA to NPR1 is direct. Our results have provided a novel evidence to support previous studies showing that NPR1 does not interact with TGA2 in untreated cells because of binding between the NT and CT of NPR1 whereas the BTB/POZ domain of NPR1 interacts with N-terminal repression domain of TGA2 upon SA treatment due to the disruption of binding between the NT and CT of NPR1. SA acts as a competitor that frees the BTB/POZ domain from its association with the NPR1.

Even though our results from gel filtration showed that Arabidopsis NPR1 may function as an SA receptor (Figure 6), these results are less convincing because they were obtained with a mutated version of NPR1 compromised in the core of the BTB/POZ domain that may affect dimerization. Although this Ala-substituted mutant is necessary to obtain a sufficient amount of soluble protein to perform the gel filtration experiment, it may cause the binding of SA to NPR1 to be less stable.

However, when comparing the results of the plant two-hybrid using a wild-type BTB/POZ domain to those using the mutated BTB/POZ domain, we found that the obtained results were similar, suggesting that the mutation of the core of BTB/POZ domain by alanine substitution does not affect the interaction with the CT despite the fact that this mutation has been found to affect dimerization.

By gel filtration analysis, it was determined that NPR1 functions as an SA receptor (Figure 8). With respect to our results from gel filtration, it is difficult to make the conclusion that the binding of SA to NPR1 is direct or indirect, which remains to be further determined. Previous studies have reported the structures of SA-binding proteins including SABP2, which is one of the well characterized SA-binding proteins. Crystallographic analysis showed that SA is bound in the active-site pocket of SABP2, located in the C-terminal end, via hydrogen-bonding, polar and van der Waals interaction. The carboxylate group of SA is bound deepest in the pocket of SABP2, and one of its oxygen atoms is hydrogen-bonded to the side chain of the histidine in the catalytic triad with the phenyl ring of SA located in a highly hydrophobic environment (Forouhar *et al.* 2004). It is speculated that SA binds to Arabidopsis NPR1 via a similar mechanism, but further investigations are required before drawing any conclusion about it.

The gel filtration analysis revealed that NPR1 eluted as an oligomer without SA. Since it has been found that BTB/POZ domain of NPR1 interacts with the CT of NPR1 (Figure 5), the oligomerization of NPR1 can be explained by two possible mechanisms. One model invokes an intermolecular interaction between the BTB/POZ

domain and CT of NPR1, and the other one a combination of intramolecular and intermolecular interactions as illustrated in the model in Figure 10. Upon SA treatment, NPR1 eluted as a dimer (Figure 8). Since the NPR1 mutant used in gel filtration has been reported to affect dimerization, it is postulated that the dimerization of NPR1 could not be through the BTB/POZ domain but could be through the CT of NPR1 (Figure 10). However, future experiments are required to confirm this hypothesis. A previous study has reported a similar effect of SA on the protein MarR, which exists as a dimer. Salicylate binds to two sites on each subunit of the MarR dimer via hydrogen bonds due to the presence of the hydroxyl and carboxyl group of SA. In each of the sites, the salicylate ring sits over a hydrophobic side chain in a pocket (Aleksun, et.al. 2001). This study of MarR has provided a new way to explain the binding of SA to NPR1 as a dimer.

One study by Tada et al. demonstrated that NPR1 fused with the green fluorescent protein (NPR1-GFP) is oligomerized in the cytosol and is reduced to a monomer when treated with DTT. The monomer enters the nucleus to activate *PR* genes (Tada et al., 2008). This finding that NPR1-GFP resides in cytosol as an oligomer is less convincing since GFP is capable of self assembling (Cabantous et al., 2005). Compared to the results showing that DTT converts the NPR1-GFP oligomer to a monomer, our results indicate that DTT has no effect on NPR1 conformation. One possibility to explain this discrepancy could be that other proteins may be present and participating in the reduction of the NPR1-GFP with DTT when analyzed in a cell-free assay, but not in our case in which a purified NPR1 is analyzed by gel

filtration.

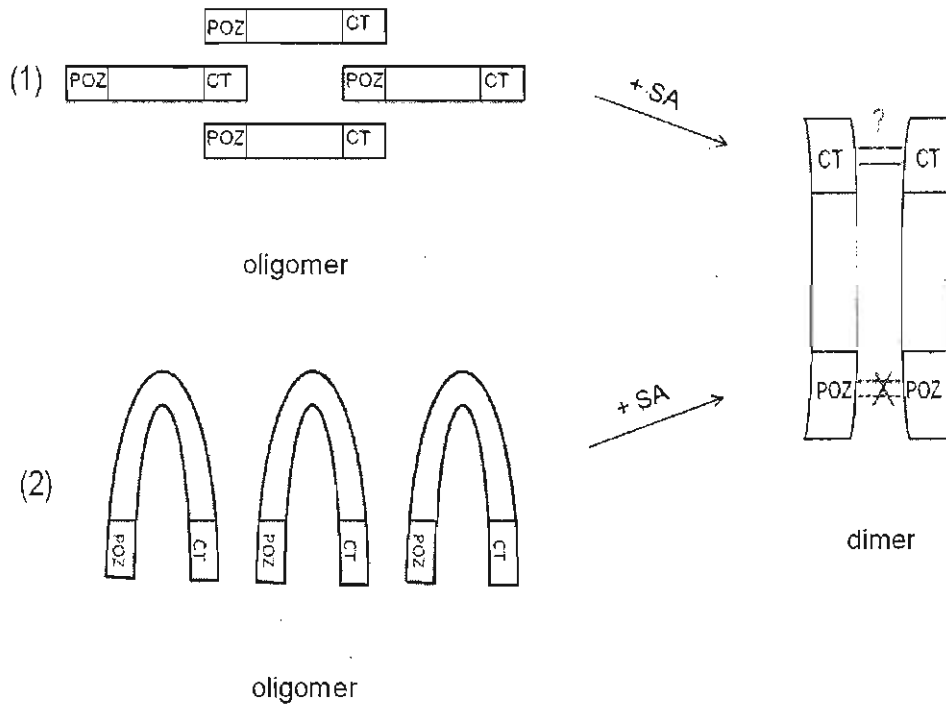


Figure 10. A proposed model showing the oligomerization of NPR1 without SA treatment and dimerization of NPR1 with SA treatment. Two possibilities are indicated for the oligomerization of NPR1 through different mechanisms.

The result in the top panel of Figure 9 showed that $\Delta 513$ fused with an HA-tag was collected in the flow-through as well as in the elution fractions. According to this result, it is difficult to make any conclusion on whether this protein binds to Ni-NTA strongly or to the NTA matrix when Ni^{2+} is absent. It is also difficult to conclude whether the high concentration of imidazole (1M imidazole was used in our experiment) can sufficiently displace high amount of $\Delta 513\text{HA}$ off the Ni-NTA even though it is demonstrated that high concentrations of imidazole are effective for

de-binding of 6XHis-tagged proteins from Ni-NTA (Qiagen) due to its similar structure with histidine. One possibility may be that the high concentration of imidazole may not be sufficient to elute all of the $\Delta 513$ HA off the Ni-NTA if the binding affinity of $\Delta 513$ HA to Ni^{2+} is high enough. Our results indicated that the addition of SA disrupted the interaction of the BTB/POZ domain with $\Delta 513$ (Figure 5 and 6), so we investigated whether SA can be used to elute $\Delta 513$ HA off the Ni-NTA. Compared to the result obtained in Figure 9, higher amount of $\Delta 513$ HA was detected in elutions (approximately 25 X higher) when 500 mM of SA was added in the elution buffer (data not shown), indicating that more $\Delta 513$ HA could be eluted off the Ni-NTA with SA. This result suggests that 1M of imidazole is not strong enough to break the interaction of all the $\Delta 513$ HA with Ni^{2+} compared with SA. However, the use of SA with a concentration of 500 mM could be too high to stabilize the interaction of $\Delta 513$ HA with Ni-NTA since it may disrupt the structure of $\Delta 513$ HA, thereby resulting in a chaotropic effect. Therefore, the use of a lower concentration of SA would be more meaningful in order to examine the effect of SA on binding of $\Delta 513$ HA to Ni-NTA.

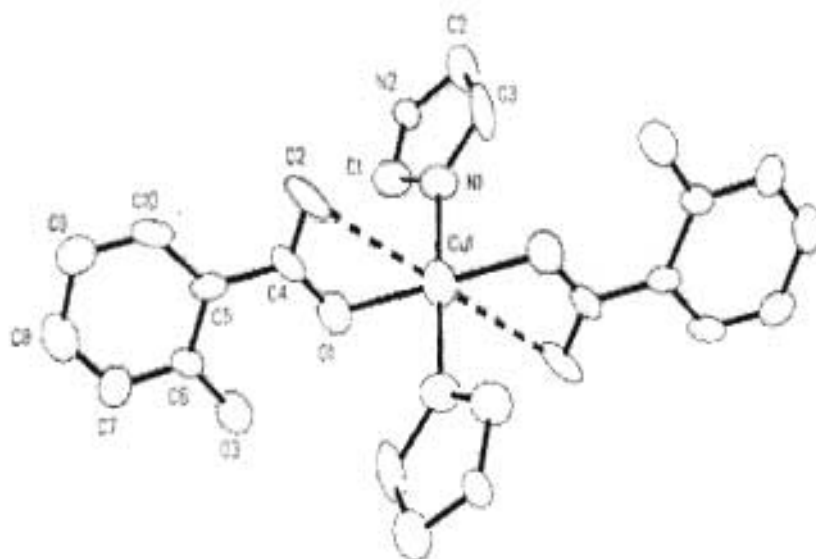
Our results indicated that the N-terminal deletion $\Delta 513$ is able to bind to Ni^{2+} in an EDTA-sensitive manner and so, functions as a metal-binding protein (Figure 9). The importance of the two Cys in $\Delta 513$ for this binding was also reported since an N-terminal deletion $\Delta 533$ lacking two Cys did not bind to Ni^{2+} and mutation of two Cys abolished the binding of $\Delta 513$ to Ni^{2+} . Previous studies have demonstrated that several metal-binding proteins including ATFP3, which can bind Ni^{2+} and a variety of

other transition metals, for example, bacterial MerP protein, a mercury ions binding protein, and Arabidopsis SBP1, a cadmium binding protein, all contain the important metal-binding motif CXXC and all participate in metal homeostasis (Sahlman and Skarfsted, 1993; Dykema et al., 1998; Dutilleul et al., 2008). The ability of $\Delta 513$ to bind Ni^{2+} could be explained by the presence of a similar metal-binding domain CXXC within the protein in position 521 to 529 as shown in Figure 4B. The finding that metal-binding proteins bind metals via a core motif CXXC provides evidence to support our results showing that two Cys are important for metal binding. It is also consistent with previous observation showing that Cys are important for metal binding in the context of zinc finger protein. In order to understand the role of NPR1 as a metal-binding protein, it will be necessary to determine the ions to which NPR1 is able to bind in vivo.

One of the metal-binding proteins SBP1 was found to be overexpressed in response to oxidative stress, and to be involved in redox control of target proteins due to the presence of the CXXC metal-binding motif (Dutilleul et al., 2008). Similarly, NPR1 is also involved in redox processes. It resides in cytosol as an oligomer maintained through intermolecular disulfide bonds whereas after SA treatment, the NPR1 oligomer is reduced to monomers which translocate to the nucleus where they interact with TGA transcription factors to activate *PR* genes and SAR (Kinkema et al., 2000; Mou et al., 2003). These findings suggest that some of the metal-binding proteins may function similarly. In fact, metal-binding proteins have been reported to be involved in metal-ion transport, metal detoxification processes and metal-resistant systems

(Dykema et al., 1998; Permina et al., 2006; Dutilleul et al., 2008). The discovery of NPR1 as a novel metal-binding protein has provided a new insight into the role of metal-binding proteins involved in plant defense systems. However, the role of NPR1 as a metal-binding protein remains to be further confirmed in future.

Since one study reported that SA is able to coordinate with metals such as copper through its carboxylate groups as shown in Figure 11, one can speculate that the binding of SA to NPR1 could be metal-dependent. However, more evidence is required to confirm this hypothesis.



[Cu(Im)₂(sal)₂]

Figure 11. A model showing the binding of SA to a metal (Abuhijleh and wood, 2001). Two salicylic acids on the left and right coordinate with copper in the centre and two imidazoles on the top and bottom also coordinate with this copper. Cu, Im, and sal indicate copper, imidazole and salicylic acids, respectively.

Chapter 5. Conclusions

In summary, we have shown that the BTB/POZ domain of the Arabidopsis NPR1 interacts with $\Delta 513$ at the CT of NPR1 by using in vivo plant two-hybrid assays and also in vitro pull-down assays. This interaction has been found to negate the function of the transactivation domain at the CT of NPR1. However, upon treatment with SA, this interaction is abolished through a mechanism which remains unknown. Furthermore, the conformation of full-length NPR1 is affected by SA when tested by gel filtration, suggesting that Arabidopsis NPR1 is an SA receptor. Further analysis by gel filtration shows that NPR1 exists as a dimer as well as an oligomer upon SA treatment. Strikingly, it has been found that $\Delta 513$ at the CT of NPR1 functions as a metal-binding protein by using pull-down assays and also that two Cys residues are important for the binding of $\Delta 513$ to metal.

Chapter 6. Future experiments

Even though examples of small molecule receptor, such as the estrogen receptor, are well understood nowadays, little is known about the function of the Arabidopsis NPR1 as a SA receptor. Thus, future work should focus on the examination of NPR1 as a SA receptor to see if the binding of SA to NPR1 is direct or indirect. To accomplish this, surface plasmon resonance (SPR) can be used to investigate the binding of SA to NPR1. The binding affinity of NPR1 with SA will be evaluated by a Biacore instrument. In SPR assay, NPR1 will be immobilized on the sensor chip with an active biological activity. Subsequently, running buffer containing SA will be injected to see whether SA directly binds to NPR1. In addition, radioactive tests are also suitable for the determination of the binding of SA to NPR1. Initially I would add SA labeled with radio-active ^{14}C to NPR1 fused with a Strep-tag which is bound to Strep-tactin column. And then I would wash excess SA and finally take the beads and count the concentration of NPR1-SA complex by using scintillation counter. The binding affinity of NPR1 with SA can be determined once the dissociation constant is estimated while the concentration of NPR1 and SA can be calculated based on Bradford and radio-activity, respectively. More studies should be performed to provide more evidence for the finding that NPR1 is a novel metal-binding protein. Diverse metal ions including Zn^{2+} , Cu^{2+} , Ca^{2+} can be used to investigate whether this metal-binding protein can bind other metals in addition to Ni^{2+} . I would use NTA column and charge it with different metal ions separately, and do the similar experiments as what I did for Ni^{2+} using $\Delta 513$ by pull-down assays.

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